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Postmortem photonic imaging of lux-modified *Salmonella* Typhimurium within the gastrointestinal tract of swine after oral inoculation in vivo[1]

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ABSTRACT: The study objective was to monitor *Salmonella* progression by photonic detection through segments of the gastrointestinal tract after oral inoculation. Pigs (∼80 kg) were inoculated orally with 3.1 or 4.1×10^{10} cfu of *Salmonella* Typhimurium transformed with plasmid pAK1-lux for a 6-h (n = 6) or 12-h (n = 6) incubation in vivo and then were killed for tissue harvest. Intestinal regions (duodenum, jejunum, ileum, large intestine) were divided into 5 replicates of 4 segments (5 cm) each for imaging. For each replicate, n = 2 segments of each region were intact, whereas n = 2 segments were opened to expose the digesta. Subsamples of digesta were analyzed to determine actual colony-forming units, and images were analyzed for relative light units per second. At 6 h, a greater ($P <$ 0.05) concentration of emitting bacteria, and consequently a greater ($P <$ 0.05) detection of photonic emissions, was observed in the small intestine than in the large intestine. The correlations (6 h) of photonic emissions in exposed segments to bacterial colony-forming units were r = 0.73, 0.62, 0.56, and 0.52 ($P <$ 0.05) in duodenum, jejunum, ileum, and large intestine, respectively. Photonic emissions were greater ($P <$ 0.05) in intact jejunum, ileum, and large intestine than in the duodenum after a 6-h incubation. At 12 h, a greater ($P <$ 0.05) concentration of emitting bacteria in jejunum and ileum of exposed segments was observed than in duodenum and large intestine of exposed segments. Photonic emissions were greater in ileum than duodenum, jejunum, and large intestine of exposed segments ($P <$ 0.05). The correlations (12 h) of photonic emissions in exposed segments to bacterial colony-forming units were r = 0.71 and 0.62 for jejunum and ileum, respectively ($P <$ 0.05). At 12 h, a greater ($P <$ 0.05) concentration of emitting bacteria in jejunum and ileum of intact segments was observed than in duodenum and large intestine. These data indicate that colony-forming units of introduced bacteria remained greater in the small intestine after 6- and 12-h incubations; we have determined that a minimum of 2.0×10^5 cfu generates detection through these tissues (∼1.0 to 21.0 relative light units/s). This study demonstrates the feasibility of using biophotonics in research models ex vivo for monitoring the pathogenicity of *Salmonella* in swine, in place of, or in conjunction with, traditional microbiological assessments and whether a greater level of sensitivity of detection and correlation to actual bacterial concentrations can be achieved.

Key words: biophotonics, gastrointestinal tract, *Salmonella* Typhimurium, swine

INTRODUCTION

The US Food Safety and Inspection Service applied regulations in 1996 to meat and poultry establishments concerning pathogen reduction (i.e., the Hazard Analysis and Critical Control Point System). Within these rules and regulations, regular microbial testing by abattoirs must be conducted to verify effective controls involving prevention and removal of fecal contamination and bacteria, and standards created to reduce *Salmonella* within the establishment (Federal Register, 1996). Participating public health laboratories in 2004 reported a total of 35,661 *Salmonella* isolates, and, based on 2004 US census population figures, the national rate was 12.1 *Salmonella* isolates per 100,000 people (CDC, 2005). Accounting for 43% of isolates, the 3 most common serotypes of *Salmonella*, as reported in 2004, were Typhimurium, Enteritidis, and Newport (CDC, 2005).

To achieve a greater understanding of the pathogenicity of *Salmonella* in swine pre- and postharvest, new models are needed that may lead to improved prevention of bacterial contamination during harvest.

Biophotonic paradigms for in vivo gastrointestinal pathogenicity models have been evaluated previously in mice and neonatal pigs (Willard et al., 2002; Burns-Guydish et al., 2005). Also, in vitro biophotonic studies have evaluated improved imaging of lux-modified bacteria (*Salmonella*) through tissue (i.e., swine skin) using optical clearing agents (Moulton et al., 2006); further attempts at monitoring *Salmonella* progression by photonic means have been achieved in larger swine models ex vivo (Moulton et al., 2006). The objective of this study was to monitor *Salmonella* progression by photonic detection through different segments of the gastrointestinal tract of swine after oral inoculation.

MATERIALS AND METHODS

These studies were approved by the Mississippi State University Institutional Animal Care and Use Committee and were conducted in accordance with the Guidelines for the Care and Use of Agriculture Animals in Agricultural Research and Teaching (FASS, 1999).

Twelve commercial-type pigs ($\sim$80 kg) were provided ad libitum access to water, and a pelleted feed (CP: 16%, Sow & Pig, Cargill Animal Nutrition, Minneapolis, MN) was provided in accordance with NRC (1998) guidelines. Pigs were inoculated orally with 20 mL of Luria-Bertani (**LB**) broth + ampicillin (**AMP**) containing either 31 (n = 6) or 41 (n = 6) billion colony-forming units of *Salmonella* Typhimurium (ATCC No. 14028; Manassas, VA) that was transformed with the plasmid pAK1-lux (Moulton et al., 2006) for a 6- (n = 6) or 12-h (n = 6) incubation in vivo. The plasmid (11,904 bp) is a broad-host-range cloning vector that is maintained at a medium copy number, is mobilizable, is compatible with numerous plasmid replicons, and contains the lux operon (Karsi et al., 2006). The lux operon is a cluster of genes (lux CDABE) isolated from a nematode symbiont bacterium *Xenorhabdus luminescens* (luciferase; Frackman et al., 1990). This operon also encodes the biosynthetic enzymes for the proper substrate. A colony of *Salmonella* Typhimurium was used to inoculate 5 mL of sterile LB broth and grown with vigorous aeration overnight at 37°C. A flask containing 50 mL of LB broth was then inoculated with 1.5 mL of bacteria in LB broth from an overnight inoculum and grown with vigorous aeration at 37°C until an optical density reading at 550 nm of 0.8 was reached using a spectrophotometer 8453 (Agilent Technologies, Palo Alto, CA). This, as determined from culture on Brilliant Green (**BG**; Becton, Dickinson and Company, Sparks, MD) agar plates, equates to $\sim$1 $\times$ 10^7 cfu of bacteria. The flask of cells was maintained on ice for 15 min until harvesting. Cells were harvested by centrifugation at 1,700 $\times$ *g* for 10 min at 4°C. The cell pellet was then resuspended in washing buffer (25 mL of sterile ice-cold

10% vol/vol glycerol), after which a sequence of 3 cycles of centrifugation and resuspension in washing buffer was conducted. The electroporation apparatus (Gene Pulsar II, Bio-Rad, Hercules, CA) was set to 2.5 kV, 25 microfarads, and 200 ohms. Forty microliters of competent cells was added to an ice-cold 2-mm gap cuvette with 1 µL of plasmid. The cuvette was loaded into the instrument chamber and the pulse was applied. The cuvette was removed and 450 mL of LB broth was added and mixed by inversion. The cells were transferred to a 1.5-mL Eppendorf tube and incubated for 1 h with moderate shaking at 37°C. After incubation, 200 to 500 µL of transformation culture was plated on BG + AMP agar plates for selection.

A limiting factor in using biophotonics for in vivo studies is the stability of the plasmid in transformed bacteria through subsequent generations without antibiotic selective pressure. When a measured quantity of photonic bacteria is inoculated in animals, an accurate measurement of the quantity of bacteria emitting photonic light across the time course of the experiment would be desirable when antibiotics are not present for selective pressure to maintain the photonic capabilities of the inoculum, as reported in the present investigation. To this end, our laboratory has previously evaluated *Salmonella* Typhimurium-pAK1-lux for stability without antibiotic selection, and the results after 0, 6, and 14 d indicated a decrease from 100% to 55% to 20% of bacteria emitting photonic light, respectively (Williams et al., 2006). Thus, for this initial investigation of photon-based detection of *Salmonella* within the gastrointestinal tract of swine, we chose to work within a 24-h time frame to maximize photonic detection and continuity between introduced bacterial concentrations and photon-emitting populations.

Each pig was killed with pentobarbital sodium (390 mg/mL) and phenytoin sodium (50 mg/mL; B-euthanasia-D Special solution, Schering-Plough Animal Health Corp., Union, NJ) administered at a concentration of 1 mL/4.5 kg intravenously into a jugular vein) after 6- or 12-h in vivo incubations postinoculation. The external body was cleaned with bleach (90%) before necropsy to remove any external bacterial contamination to prevent inadvertent transfer of shed photon emitting bacteria (i.e., from fecal matter on skin) to the internal compartment upon postmortem examination. The small and large intestines were removed and divided into 5 replicates of 4 segments (5 cm) each [duodenum, jejunum, ileum, and large intestine (proximal and distal collectively)] for imaging. The detection of photonic emissions was acquired using a Stanford Photonics imaging system (XR/MEGA-10Z; Stanford Photonics Inc., Palo Alto, CA) using low gain and 1 $\times$ 1 binning with an acquisition time of 10 min. For each replicate, n = 2 segments of each region remained intact, whereas n = 2 segments were cut open on 1 side to expose the digesta for comparisons of intact versus exposed segments (i.e., removing the intestinal wall as a barrier to photonic detection). After imaging, two 10-µL loop subsamples

of digesta were collected from every segment and serially diluted in 1 mL of LB + AMP. The dilutions were plated on BG + AMP agar and incubated overnight (37°C). The plates were counted for total colony-forming units and then imaged to measure the percentage of photon-emitting colony-forming units in relation to the total mixed bacterial colonies cultured.

The photonic images of the intestinal segments were analyzed by measuring a uniformed rectangular area encapsulating the center of each segment for relative light units (**RLU**) per second using Image J software (National Institutes of Health, Bethesda, MD) and were reported after background (a nonsegment area of the image) subtraction (Figure 1). For statistical analysis, photonics from intestinal segments and bacterial concentrations were analyzed using GLM and least significant means (SAS Inst. Inc., Cary, NC) to determine differences among intestinal types. Correlations were performed between quantified photonic emissions and bacterial concentrations using Fisher's LSD.

RESULTS

The 6-h incubation resulted in greater ($P < 0.05$) concentrations of emitting bacteria, and consequently greater ($P < 0.05$) detection of photonic emissions, in exposed segments of the small intestine (duodenum, jejunum, and ileum) than in the large intestine (Table 1). Positive correlations were observed between photonic emissions in exposed segments and the subsample of bacterial concentrations ($P < 0.05$) in duodenum, jejunum, ileum, and large intestine (Table 1). The 6-h incubation resulted in a greater ($P < 0.05$) concentration of emitting bacteria, and consequently greater ($P < 0.05$) detection of photonic emissions, in intact segments of the small intestine (duodenum, jejunum, and ileum) than in the large intestine (Table 1). The photonic emissions of intact segments were correlated to the subsample bacterial concentration ($P < 0.05$) for duodenum but not ($P > 0.05$) for jejunum, ileum, and large intestine (Table 1).

The 12-h incubation resulted in greater ($P < 0.05$) concentrations of emitting bacteria in the jejunum and ileum of exposed segments than in the duodenum and large intestine (Table 1). The detection of photonic emissions was greater in the ileum than the duodenum, jejunum, and large intestine of exposed segments (Table 1). Positive correlations were observed between photonic emissions in exposed segments and the subsample of bacterial concentrations ($P < 0.05$) in the ileum and jejunum, but not ($P > 0.05$) for duodenum and large intestine (Table 1). The 12-h incubation resulted in greater ($P < 0.05$) concentrations of emitting bacteria in the jejunum and ileum of intact segments than in the duodenum and large intestine (Table 1). The detection of photonic emissions was greater ($P < 0.05$) in the jejunum, ileum, and large intestine than in the duodenum of intact segments (Table 1). The photonic emissions in intact segments correlated to the sub-

sample bacterial concentrations for jejunum and large intestine ($P < 0.05$) but not for the duodenum and ileum ($P > 0.05$) after 12-h incubation of inoculums in swine (Table 1). Data from the present study (Table 1) indicate that bacterial concentrations were greater in the small intestine and that a minimal result for this experiment of 2.0×10^5 cfu will generate detection of photonic emissions through the intact small intestinal wall of ~1.0 to 21.0 RLU/s after a 6-h incubation in the pig. After a 12-h incubation, bacterial concentrations were greater in the lower portion of small intestine, and a minimal result for this experiment of 1.6×10^7 cfu will generate detection of photonic emissions through the intact small intestinal wall of ~1.5 to 5.0 RLU/s. An example image of the photonic emissions detected in digesta from swine gastrointestinal segments of ileum and duodenum postinoculation is represented with a color overlay (Figure 1).

DISCUSSION

The FoodNet Surveillance Report for 2004 stated that 5% of the 6,498 *Salmonella* cases for the US population were identified as being outbreak-related. Seventy-eight percent of the outbreak-associated cases were food-related (CDC, 2006). Therefore, for the public safety, nontyphoidal qualitative microbial testing has been mandated in the food-processing industry by USDA and Food and Drug Administration regulations. However, traditional microbiological methods involve enriching the food sample and performing various media-based metabolic tests (agar plates or slants). These typically require 3 to 7 d to obtain a result. More rapid-screening tests based on immunochemical or nucleic acid technologies have been developed and can provide results in 8 to 48 h. However, results from screening tests are considered presumptive by the USDA (USDA-FSIS, 1998). Others have used traditional microbiological methods for evaluating on-farm prevalence levels, and the estimated costs were calculated to be $11.25 per pig (Gorton et al., 1997). Other on-farm research gives a range of $1.76 to $4.72 per pig depending on the method just for sampling costs and does not include the laboratory procedures. This type of testing program may require monitoring or verification of testing information; thus, it is unclear whether the implementation of on-farm *Salmonella* testing will significantly contribute to reducing *Salmonella* in the pork supply (Mark et al., 1999). Biophotonic technology may not be directly applicable to on-farm or consumer product testing but could improve analysis of bacteria in experimental research models aimed at evaluating testing standards and effectiveness.

Research establishments use the same standard bacteriological techniques for examination of feces to monitor transmission of *Salmonella* in pigs as well as utilizing *Salmonella*-ELISA to screen pigs for serological evidence of an infection (Van Winsen et al., 2001). The following are some research examples of standard bac-
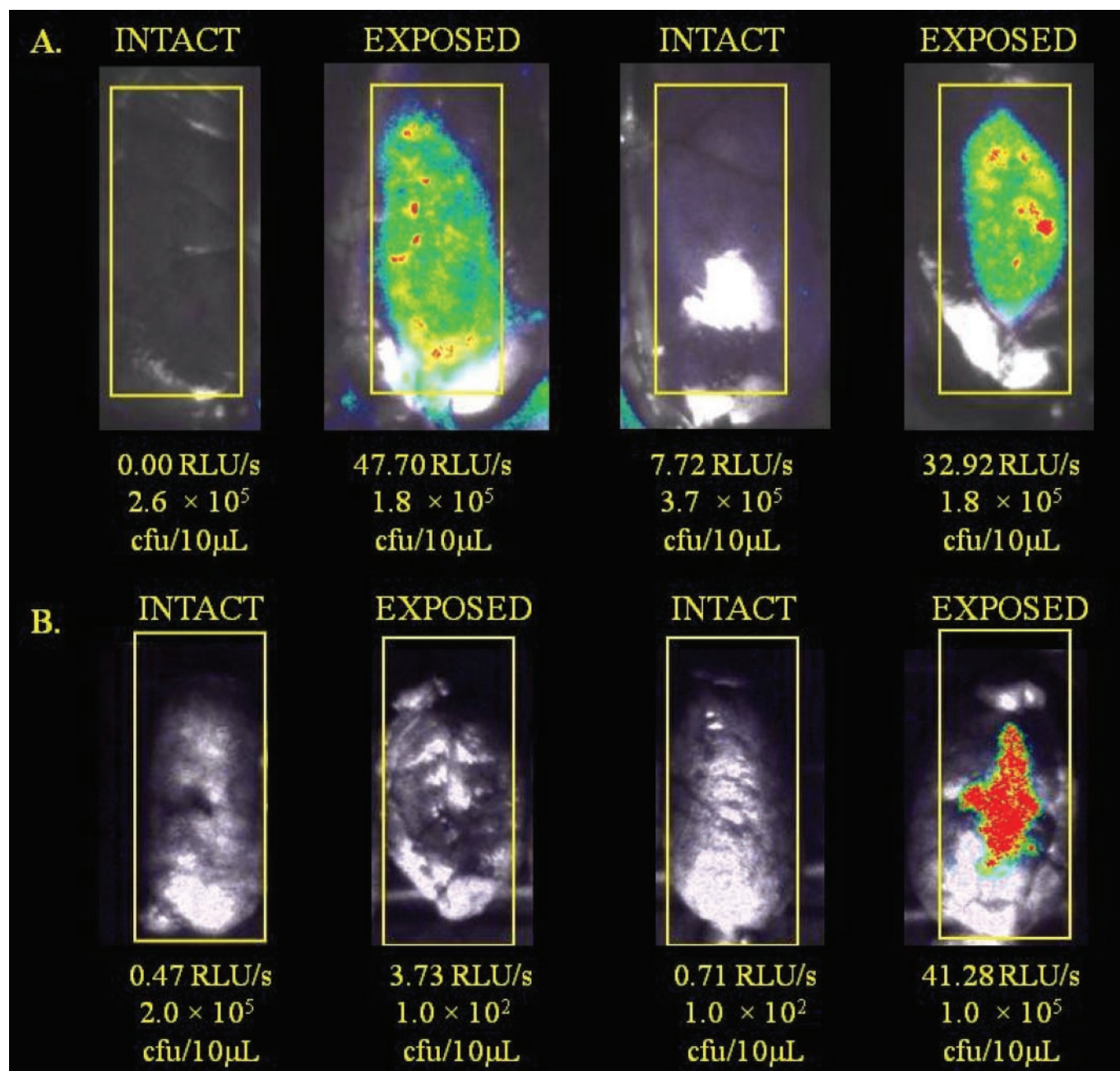
Figure 1. A representative color overlay of intact (closed) and exposed (opened) gastrointestinal segments containing digesta from swine ileum (panel A) and duodenum (panel B). Rectangular area boxes are representative of the areas used for photonic emission measurements during a 10-min acquisition as detected by a Stanford Photonics XR/MEGA-10Zero camera (Stanford Photonics Inc., Palo Alto, CA). RLU = relative light units.

teriological techniques used in experiments. After clinical salmonellosis was observed in a swine herd, *Salmonella* Typhimurium was found in feces for 14 d and in extraintestinal tissues for as many as 7 d postexposure when using PCR assay and bacteriological culture (Cote et al., 2004). Using standard culture methods applied to *Salmonella* detection in food during pork processing, the greatest values of *Salmonella* occurrence were achieved in the ileocolic lymph nodes (18.8%) and in the ileum (13.9%; Vieira-Pinto et al., 2005). Moreover, it has been determined by scanning and transmission electron microscopy that cellular invasion by *Salmonella* Typhimurium is nonspecific, but rapid, in swine

ileum (Meyerholz et al., 2002). Although the present study was limited to a 12-h period of exposure, at the 12-h time point, the concentrations of bacteria and detected photonic emissions within intact and exposed segments were greater in the jejunum and ileum than in other segments. Using postoral challenge of mice with *Salmonella* Typhimurium and standard bacteriological practices, it was determined that infection developed in the Peyer's patches of the small intestine within 3 h and that the concentrations of bacteria found in the cecum were in the cecal contents and not the cecal wall (Hohmann et al., 1978). It was determined that several epithelial cell types were invaded by *Salmonella* and that

Table 1. Mean concentration (±SE) of emitting *Salmonella* Typhimurium-pAK1-lux and photons detected from harvested gastrointestinal tissues (intact and digesta exposed) at 6 and 12 h after oral inoculation in swine

Item	Intact			Exposed		
	Emitting concentration, cfu	Photonic emissions, RLU[1]/s	r	Emitting concentration, cfu	Photonic emissions, RLU/s	r
6 h[2]						
Duodenum[3]	$3.2 \times 10^6 \pm 8.6 \times 10^{5,a}$	21.2 ± 4.0^a	0.62*	$3.8 \times 10^6 \pm 9.9 \times 10^{5,a}$	32.2 ± 5.0^b	0.73*
Jejunum[3]	$1.2 \times 10^6 \pm 5.1 \times 10^{5,b}$	11.0 ± 3.0^{cd}	0.19	$9.5 \times 10^5 \pm 2.6 \times 10^{5,b}$	24.7 ± 4.0^b	0.63*
Ileum[3]	$2.4 \times 10^6 \pm 8.9 \times 10^{5,ab}$	17.0 ± 4.0^{ac}	0.12	$3.9 \times 10^6 \pm 1.3 \times 10^{6,a}$	30.0 ± 5.0^b	0.56*
Large intestine[3]	$2.1 \times 10^5 \pm 4.7 \times 10^{4,c}$	1.2 ± 0.5^e	0.23	$2.6 \times 10^5 \pm 6.2 \times 10^{4,c}$	7.4 ± 3.0^d	0.52*
12 h[2]						
Duodenum[3]	$2.9 \times 10^4 \pm 1.1 \times 10^{4,a}$	0.2 ± 0.04^a	0.02	$4.0 \times 10^4 \pm 2.0 \times 10^{4,a}$	2.0 ± 0.8^{ab}	0.18
Jejunum[3]	$1.6 \times 10^7 \pm 1.1 \times 10^{7,b}$	1.5 ± 0.7^{ab}	0.83*	$6.5 \times 10^6 \pm 5.3 \times 10^{6,b}$	6.2 ± 2.2^{abc}	0.71*
Ileum[3]	$2.7 \times 10^7 \pm 2.6 \times 10^{7,b}$	5.1 ± 1.8^{abc}	0.07	$4.3 \times 10^7 \pm 2.8 \times 10^{7,b}$	16.1 ± 2.6^d	0.62*
Large intestine[3]	$33.5 \times 10^5 \pm 6.3 \times 10^{4,c}$	2.9 ± 0.8^{abc}	0.51*	$3.4 \times 10^5 \pm 6.2 \times 10^{4,c}$	4.7 ± 2.2^{bc}	0.26

[a–e]Values within a column or row without a common superscript differ ($P < 0.05$).

[1]RLU = relative light units.

[2]Swine inoculation ~3.1×10^{10} cfu of *Salmonella* Typhimurium-pAK1-lux (incubated 6 h) and ~4.1×10^{10} cfu of *Salmonella* Typhimurium-pAK1-lux (incubated 12 h).

[3]For each 6- and 12-h intact or exposed intestinal segment, the sample size was n = 60.

*Asterisks denote significant r values ($P < 0.05$).

Peyer's patches represent the main portal of entry in early *Salmonella* infection of swine using a jejunal loop model and immunocytochemistry methods (Schauser et al., 2004). Using a ligated intestinal loop model in mice, *Salmonella* Typhimurium initiated infection by penetrating the intestinal epithelium and preferentially interacting with Peyer's patches (Jones et al., 1994). These experimental methods are intense (48 to 96 h) and labor-intensive for developing models of pathogenicity to determine actions that would potentially be implemented in Hazard Analysis and Critical Control Point programs from the farm to the abattoir. Similar to the above studies in mice and swine, our study determined that within 6 h, an infection developed in the small intestine (duodenum, jejunum, and ileum) with greater concentrations of bacteria in the small intestine than in the large intestine using standard microbiological techniques. When evaluated after an increased time (12 h) of incubation in swine, our study resulted in greater concentrations in the jejunum and ileum than the duodenum and large intestine. However, from the use of bioluminescent *Salmonella* in our study, positive correlations between photonic emissions (10-min acquisition time) and bacterial concentrations were observed for exposed segments of intestine after 6-h postoral inoculation. Correlations of photonic emissions in intact segments were for duodenum only after 6-h postoral inoculation. Positive correlations were observed between photonic emissions in the exposed ileum and jejunum but not for exposed duodenum and large intestine after 12-h postoral inoculation. Correlations of photonic emissions in intact segments were significant for the jejunum and large intestine after 12-h postoral inoculation but not for duodenum and ileum. The reduction in positive correlations for intact segments is due to the tissue barrier causing photonic scattering and may be improved by using optical clearing agents (Moulton et al., 2006).

This study was conducted to monitor *Salmonella* progression by photonic detection through different segments of the gastrointestinal tract of swine after oral inoculation for the potential of creating a research model that may quantitate bacterial invasion more quickly, efficiently, and definitively in the swine gastrointestinal tract. Researchers can better understand disease pathology, pharmacokinetics, and other biomolecular processes taking place in the living animal by imaging the whole animal at multiple time points as opposed to euthanizing subsets of animals at various time points to collect tissues for analysis (Contag, 2002). This application can improve statistical quality of data because each experimental animal is its own statistical control and experimental variability can be reduced by more rapid and computerized in vivo data collection using imaging technologies (Contag, 2002). Although the present study, by design, involved time-dependent reductions of swine for data-point determinations, this study was aimed at the development of a live animal model for swine using multiple time point determinations of *Salmonella* movements in vivo. This has been reported previously by our group for neonatal swine using biophotonic approaches (Willard et. al., 2002) and as part of the present study is being modified for feeder and market weight swine research applications. Similar research applications to our *Salmonella* swine model have been conducted by others and are listed below. Luciferase-encoded bacterial and green fluorescent protein as a dual-marker system was used for monitoring specific bacteria in situ in environmental soil samples (Unge et al., 1999). *Haemophilus influenzae* infections were monitored within the nasopharynge, eustachian tubes, and middle ears of chinchillas after

intranasal and transbullar challenges with biophotonic transformed isolates (Novotny et al., 2005). Susceptibility to infection with oral introduction of bioluminescent *Salmonella* Typhimurium in young mice compared with adult mice was evaluated using in vivo bioluminescence imaging to monitor progression of infection over a 2-wk period (Burns-Guydish et al., 2005). *Staphylococcus aureus* infections using bioluminescence were monitored in vivo noninvasively and with sensitivity, as well as requiring fewer animals than conventional methodologies when determining effectiveness of antibiotic treatments in mice that had been injected in the thigh muscle with bioluminescent *Staphylococcus aureus* (Francis et al., 2000). Therefore, our study, along with these other studies, continues to perpetuate the idea that biophotonic techniques could potentially improve development of bacterial pathogenicity models.

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